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Immunohistochemical detection of axillary lymph node micrometastases in node negative breast cancer patients using cytokeratin and epithelial membrane antigen

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Abstract

Background and Objective: The study was conducted to detect occult metastases in lymph node negative breast cancer patients using cytokeratin (CK) and epithelial membrane antigen (EMA) immunohistochemistry (IHC) and correlate this with primary tumor size and grade. **Materials and Methods:** A total of 32 cases including 12 prospective and 20 retrospective cases of axillary lymph node negative breast cancer were studied. CK and EMA IHC were performed to detect micrometastases. **Results:** Axillary lymph node metastases were detected in 18.75% of previously node negative cases using CK and EMA IHC. CK was found to be more sensitive for detection of metastases compared to EMA. A highly significant correlation was observed between tumor grade and axillary lymph node metastases detected by CK and EMA. However, no significant correlation was found between tumor size and axillary lymph node metastases detected by IHC. **Conclusion:** In the present study, there was an increase of 18.75% in the occult metastases detection rate using CK and EMA. To conclude, IHC detection of occult metastases should be done using CK in all axillary node negative cases, especially in T1 and T2 stage tumors.

Key words: Axillary node, breast cancer, cytokeratin, epithelial membrane antigen, micrometastases

Introduction

Breast cancer is one of the commonest cancers among women in India.[1] Prognosis in patients with carcinoma breast depends predominantly on size and histological grade of the tumor and the extent of lymph node involvement. Lymphatic metastases in carcinoma breast occur mainly to the axillary and internal mammary group of lymph nodes.[2] Sentinel node is the first node in the regional lymphatic system that drains the primary tumor. If it is uninvolved, the other nodes are unlikely to bear metastases.[3]

Micrometastasis has been defined, according to the latest revision of TNM classification system as metastasis more than 0.2 mm in size, but less than 2 mm. If metastasis is more than 2 mm in size it is referred to as macrometastasis.[4] The accuracy of detecting micrometastases in the pathology laboratory has been a major concern. A variety of methods including multiple level sectioning and immunohistochemistry (IHC) on paraffin blocks have been shown to be superior to the original single hematoxylin and eosin (H and E) staining in presenting or exposing micrometastases. Sections from cases in which axillary lymph node had been reported free of metastases were stained using monoclonal antibodies pancytokeratin AE1/AE3 and anti-epithelial membrane antigen (EMA). It was noted that there was a 7-20% increase in diagnosing metastases.[5,6]

Our study was conducted to detect axillary lymph node micrometastases in node negative breast cancer patients using cytokeratin (CK) and EMA and to correlate this with tumor size and grade.

Materials and Methods

The study included 32 cases comprising of 20 retrospective and 12 prospective cases of axillary lymph node negative patients with breast cancer (from April 2002 to March 2007). A total of 178 mastectomies were performed or breast cancer during this time frame. Out of 178, 32 (17.97%) cases had negative axillary lymph nodes on routine H and E staining and these cases were selected for the study. The study was approved by the institutional review board. Written consent was obtained from all the patients included in the study.

Two sections of 3-5 μm thickness from each block were cut at 50 μm intervals on poly l-lysine coated slides. Antigen retrieval was performed by heat induced epitope retrieval using microwave oven. IHC was performed using avidin biotin technique (using labeled streptavidin biotin (LSAB) + kit) with Dako Monoclonal Mouse anti-Human CK clone AE1/AE3 (dilution 1:50) and Novocastra NCL-EMA Mouse Monoclonal antibody (dilution 1:100).

CK staining gave brown cytoplasmic reactivity. EMA staining gave brown cytoplasmic reactivity with membrane enhancement. Cells were considered to be occult node metastases if they were: Immunoreactive (expressed either CK or EMA antigens); found within the substance of lymph nodes; and were morphologically consistent with cancer cells.

Statistical analysis

Statistical evaluation was done using Statistical Package for Social Sciences software version 14. Student's *t*-test was applied. A $P \leq 0.05$ was considered as significant.

Results

Out of 178 patients who underwent modified radical mastectomy for breast carcinoma in our institution during the study period (April 2002–March 2007), 146 (82.03%) were diagnosed as axillary lymph node positive for metastases and 32 (17.97%) were axillary lymph node negative for metastases on H and E examination. These 32 cases were taken up for the study.

Twenty-seven cases (84.4%) were diagnosed with infiltrating duct carcinoma not otherwise specified, two cases (6.3%) as medullary carcinoma and one case (3.1%) each of papillary, colloid, and metaplastic carcinoma breast. The mean age of patients was 50.84 years (range 30-70 years). Out of 32 patients with node negative breast carcinoma, 31 (96.87%) were females and only one patient was male (3.13%). None of the patients had a family history of breast cancer.

Twenty-three cases (71.86%) were stage I and II of breast cancer, seven cases (22.87%) were stage III, and only one case was stage IV with liver metastasis, but was clinically node negative. The median tumor size was 2.75 cm (range 0.5 cm-12 cm).

Tumor grading was done by Modified Bloom and Richardson Grading System. Nineteen (63.33%) were classified as grade 1, nine cases (30%) as grade 2, and two cases (6.67%) were grade 3 invasive carcinoma. Two cases of medullary carcinoma were not graded.

In ten cases (31.25%), six to 10 lymph nodes were dissected, in nine patients (28.12%), one to five nodes were dissected, six cases (18.75%) had 11-15 lymph nodes dissected, five (15.62%) had 16-20 nodes dissected, and two cases had 26-30 nodes dissected. Out of 32 cases studied, eight (25%) had sentinel lymph node identified by 1% isosulfan blue dye injection during axillary lymph node dissection and 24 (75%) did not have sentinel lymph node identified intraoperatively. This was indeed a limitation of our work as IHC staining had to be done in the remaining 24 cases (in which sentinel lymph node was not detected) on all the lymph nodes, thereby increasing the cost.

Six out of 32 cases (18.75%) were positive for lymph node metastases by CK IHC and four out of 32 cases (13%) were positive for lymph node metastases by EMA IHC. All the four EMA positive cases were also positive for CK [Table 1]. Out of these six cases, three cases had micrometastases in two lymph nodes, while three cases had micrometastases in single node. Three cases were found to have single isolated cells positive for CK, but the nuclear features were not similar to that of main tumor, so they were not considered metastatic. The sensitivity of detection of occult metastases by CK was 100% while that of EMA was 66.66%. The specificity of detection was 92.85% for CK and 100% for EMA. The positive predictive value was 1.00 for EMA and 0.66 for CK. There was a significant correlation between CK and EMA positive metastases ($P = 0.001$).

No statistically significant correlation was found between tumor size and lymph node metastases detected by CK ($P = 0.326$) or EMA ($P = 0.467$). We found a metastases detection rate of 26.66% in T2 tumors (2 cm-5 cm), 20% in T1 tumors (<2 cm), and 0 in T3 tumors (>5 cm). There was a statistically significant correlation between tumor grade (Modified Bloom and Richardson Grading System) and lymph node metastases detected by CK ($P = 0.007$) [Table 2] and between tumor grade and lymph node metastases detected by EMA ($P = 0.001$). 63.33% were classified as grade 1, 30% as grade 2, and 6.67% as grade 3 carcinoma. Hundred percent of the grade 3 tumors were positive for occult lymph node metastases by both CK and EMA. 22.22% of grade 2 tumors and 10.53% of grade 1 tumors were found to have occult lymph node metastases.

Two out of three cases (66.6%) which had two tumors in one breast were found to have CK positive metastases in lymph node while four out of 29 (13.8%) had metastases detected by CK. There was a statistically significant ($P = 0.026$) correlation between tumor number and lymph node metastases detected by CK. However, the correlation between tumor number and lymph node metastases detected by EMA was not statistically significant ($P = 0.340$).

A statistically significant correlation was found between lymph node number and lymph node metastases detected by CK ($P = 0.049$) and EMA ($P = 0.0028$). All the cases with lymph node number between 26 and 30 were found to be positive for metastases by both CK and EMA IHC (100%).

Lymph node metastases were detected by CK in four out of eight cases (50%) among whom sentinel lymph nodes were identified by the surgeon intraoperatively and only in two cases out of 24 (16.6%) in whom sentinel nodes were not identified. There was a statistically significant correlation between sentinel lymph node and metastases detection by CK IHC ($P = 0.023$) and sentinel node and metastases detection by EMA IHC ($P = 0.039$). A comparative analysis of characteristics of patients with micrometastases (detected by CK or EMA) and those without micrometastases is shown in Table 3.

In our study, all the patients in whom micrometastases were detected by either CK or EMA were given adjuvant chemotherapy similar to the other group of patients without micrometastases. However, the patients without micrometastases were kept on close follow-up for the development of axillary recurrence or distant metastases. The median follow-up was 56 months; however, two were lost to follow-up. None of the

Table 1: Correlation between lymph node metastases by cytokeratin and EMA (*P*=0.001)

Lymph node CK	Lymph node EMA negative	Lymph node EMA positive	Total
Negative	26	0	26
Positive	2	4	6
Total	28	4	32

CK: Cytokeratin, EMA: Epithelial membrane antigen

Table 2: Correlation between tumor grade and lymph node metastases by cytokeratin (*P*=0.007)

Tumor grade	Cytokeratin positive	Cytokeratin negative	Total
1	2	17	19
2	2	7	9
3	2	0	2
Total	6	24	30

Two cases of medullary carcinoma were excluded from the grading (*n*=30)

Table 3: Characteristics of patients with micrometastases and those without micrometastases

	Patients with micrometastases, detected by CK or EMA	Patients without micrometastases
No. of patients	6	26
Average no. of lymph nodes	16.5	9.3
Sentinel lymph node positivity	Nil	2/26
Margins involved by tumor	1/6	7/26
Grade of tumor	Grade 1 (2 cases); grade 2 (2 cases); grade 3 (2 cases)	Grade 1 (17 cases); grade 2 (7 cases); grade 3 (none); (2 cases of medullary carcinoma not included)
Pathological tumor size	T1 (3 cases); T2 (3 cases)	T1 (9 cases); T2 (11 cases); T3 (6 cases)
Skin involved by tumor	None	2/26

CK: Cytokeratin, EMA: Epithelial membrane antigen

patients developed recurrence or metastases and all the patients are alive till date.

Discussion

The incidence of axillary lymph node negative breast carcinoma in the present study was 17.97%, which is much less when compared to the Western literature. Colleoni *et al*.,[7] and Viale *et al*.,[8] found an incidence of 54 and 66.8% of lymph node negative breast cancer, respectively.

The sensitivity of detection of occult metastases by CK and EMA was 100 and 66.66%, respectively; while specificity of detection was 92.85% for CK and 100% for EMA. CK and EMA positive metastases showed significant correlation. This increase in detection rate of occult metastases is seen in accordance with other studies in the literature. Wells *et al*.,[6] (using CK, EMA) and Cote *et al*.,[9] (using CK) reported an increase in detection rate by 15 and 20%, respectively; while Hainsworth *et al*.,[10] and Kohlberger *et al*.,[5] found 12 and 11% increased detection of micrometastases, respectively.

In our study, metastases detection rate was found to be 26.66% in T2 tumors, 20% in T1 tumors (<2 cm), and 0 in T3 tumors (>5 cm). The observation of most of the micrometastases in T1 and T2 patients could probably be explained by the occurrence of macrometastases in patients with larger tumor size (T3) as compared to smaller tumor size (T1 or T2). Wong *et al*., found occult metastases in 50, 33, and 17 cases of breast cancer with T1, T2, and T3 tumor size, respectively.[11]

Occult lymph node metastases by both CK and EMA were detected in 100% of the grade 3, 22.22% of grade 2 tumors, and 10.53% of grade 1 tumors; highlighting the fact that with increasing tumor grade there is an increase in probability of occult micrometastases detection by immunohistochemistry. This is in concordance with Viale *et al*.,[8] who reported 22.7, 32.2, and 38% metastases detection rates in grade 1, 2, and 3 tumors, respectively. They concluded that higher tumor grades (grades 2 and 3) are associated significantly with higher prevalence of sentinel lymph node metastases.[8]

Statistically significant correlation was observed between sentinel lymph node and metastases detection by CK IHC as well as EMA IHC in the present study. Viale *et al*.,[8] detected metastases in sentinel lymph node of 33.2% breast cancer patients. The sentinel lymph node was the only lymph node involved in 57.3% of the patients. They concluded that examination of sentinel lymph node increases the detection of micrometastases and isolated tumor cells that would have been otherwise undetected.

The clinical and pathological TNM stages do not always correlate. For example, inflammatory changes in the skin of the breast do not necessarily reflect dermal lymphatic invasion (inflammatory carcinoma), and clinical estimation of the presence or absence of nodal involvement is incorrect in about one-third of the cases.[12] In our study, we found that in 16 cases (50%), the clinical TNM stage did not correlate with pathological TNM stage. In 10 cases, clinically enlarged axillary lymph nodes were negative by both H and E and IHC examination. In four cases, clinically negative lymph nodes were found to have micrometastases. Gross and clinical tumor size did not match in four cases. The Yorkshire Breast Cancer group found that pathologists and surgeons agree on tumor size

in only 54% cases.[13] The advantage of pathologic staging is in the certainty it provides regarding the true extent of the lesion in the sampled areas.[14]

In recent years, several groups have used reverse-transcriptase polymerase chain reaction (RT-PCR) to further improve the detection of lymph node metastases beyond that obtained with serial sectioning and IHC. Sakaguchi *et al*. used CK-19 and epithelial glycoprotein 2 (EGP2) RT-PCR for detection of micrometastases in breast cancer as they are expressed by most epithelial cells and cancer cells; but not by lymph node lymphocytes, peripheral blood, and bone marrow cells. Studies of bone marrow indicate that IHC can detect one breast cancer cell among 10^4-10^5 normal cells. In comparison, RT-PCR is 10-100 times more sensitive as it can detect one cancer cell in 10^6 normal cells.[15]

Recently, several studies[16,17] have evaluated and compared the utility of touch imprints, frozen sections, and CK immunostaining in intraoperative evaluation of axillary sentinel lymph nodes in breast cancer patients. Krishnamurthy *et al*.[16] devised a rapid pancytokeratin IHC staining to be used intraoperatively. The CK staining took around 25 min and they observed a sensitivity of 80% when compared to 75% for frozen section and 45% for touch imprint cytology alone.

Vincent-Solomon *et al*.[18] reviewed the various detection methods and prognostic impact of bone marrow micrometastases in breast cancer. They observed that immunocytochemistry is the most commonly used method to detect disseminated tumor cells (DTC) in bone marrow aspirates and remains the gold standard with a sensitivity ranging from 1 DTC in 10^5 to 1 DTC in 10^6 leucocytes.

Axillary lymph node metastasis is one of the most important prognostic factors in breast carcinoma. Survival rates depend not only on the presence or absence of metastases, but also on the number of nodes involved, level of axillary node, the amount of metastatic tumor, and the presence or absence of extranodal spread.[2,4] The prognostic importance of micrometastases or isolated tumor cells is still being elucidated.

To conclude, IHC detection of occult metastases in all axillary lymph node negative cases should be considered using CK especially in cases with high tumor grade, more than one tumor in the same breast, and higher lymph node number. The sentinel lymph node should be examined by CK IHC to increase the occult metastases detection rate in node negative patients, especially in T1 and T2 stage tumors. However, the prognostic significance of axillary lymph node micrometastases needs further evaluation.

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